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Calcium-Mediated Responses of Maize to Oxygen Deprivation*

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Abstract—Oxygen limitation dramatically alters the patterns of gene expression as well as development of plants. Complete removal of O_2 leads to an immediate cessation of protein synthesis followed by a selective synthesis of about twenty anaerobic proteins in maize (*Zea mays* L.) seedlings. Among these are enzymes involved in glycolysis and related processes. However, inducible genes that have different functions were also found; they may function in other, perhaps more long-term, processes of adaptations to flooding, such as aerenchyma formation and root-tip death. Our recent research has addressed two questions: how these gene expression changes are initiated and how do these responses culminate in the overall adaptation of plants to flooding-stress. The results obtained indicate that an early rise in cytosolic Ca^{2+} as well as a quick establishment of ionic homeostasis may be essential for the induction of adaptive changes at the cellular as well as organismal level.

Key words: Zea mays - oxygen deprivation - calcium - ionic homeostasis - glutamate decarboxylase - aerenchyma - root tip death - sucrose synthase - protease - xyloglucan endotransglycosylase

INTRODUCTION

Oxygen availability is the primary limiting factor for plant growth in flooded soils. The sudden excess of water due to flooding not only threatens the food supply of human populations but also affects the vegetation in river plains. During 1993 flooding, ~20 million acres of maize (*Zea mays* L.) and soybean (*Glycine max* L.) were inundated in the Midwestern United States leading to heavy economic losses, as estimated by the United States Department of Agriculture, National Agricultural Statistics Service [1]. Plants also suffer from oxygen limitation during normal plant development due to their bulky biomass and lack of a specialized circulatory system. The consequences of oxygen deficit on reproductive development were shown to be agronomically important [2] and have recently received renewed attention [3, 4].

Anaerobic treatment of maize seedlings drastically alters the profile of total protein synthesis. Under an anaerobic environment, 20 proteins, that account for more than 70% of the total translation, are selectively synthesized [5]. The coordinated synthesis of anaerobic proteins (ANPs) appears to follow the programmed transcription and co-regulation of genes that encode these proteins. This proposal [5] is supported by the recent gene expression profiling studies and analysis of upstream regulatory regions of genes that are responsive to low oxygen treatment in *Arabidopsis* root cultures [6]. Most of the ANPs identified were found to be enzymes of glycolysis or sugar–phosphate metabolism; such as aldolase [7], pyruvate decarboxylase [8, 9], enolase [10], glucose-6-phosphate isomerase [11], glyceraldehyde-3-phosphate dehydrogenase [12], sucrose synthase [13], and alcohol dehydrogenase [14]. However, some genes not involved in glucose–phosphate metabolism [6, 15–19], have also been found to be induced by anoxia.

Anaerobiosis results in alterations of gene expression in plants leading to the accumulation of the ANPs. These alterations occur at transcriptional, translational and post-translational levels [5, 20–30]. At the level of translation, anaerobic treatment of maize seedlings disrupts polysomes [31] and leads to a redirection of protein synthesis [5, 26]. In the first 5 h of anaerobic treatment (transition period), there is a rapid increase in the synthesis of a class of polypeptides (~33 kD, the transition polypeptides). After 90 min of anoxia, the synthesis of ANPs is induced. After 72 h, protein synthesis decreases concurrently with the start of seedling death [5]. The molecular basis of this selective translation is not yet fully understood. Specific structural determinants in the untranslated regions of mRNA [32] and induction of specific RNA-binding proteins [6] are important in this process. In addition, post-translational regulation of initiation factors as well as ribosomal pro-

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Abbreviations: ADH—alcohol dehydrogenase; AIP—anoxia-induced protease; ANP—anaerobic protein; $[Ca]_i$—cytosolic free Ca; CAM—calmodulin; CAP1—calcium pump; GAD—glutamate decarboxylase; PCD—programmed cell death; PMCA—plasma membrane located Ca^{2+}-ATPase; SERCA—animal Eh-located Ca^{2+}-ATPase; SH1—anaerobically-induced SS1; SS—sucrose synthase; XET—xyloglucan *endo*transglycolase.

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teins by reversible phosphorylation appears to play a role [24, 29, 33]. Other post-translational changes of proteins have also been reported to occur under anoxia [30]. From the two-dimensional electrophoresis and mass-spectroscopic analysis of proteins synthesized in the root tip under different oxygen regimes, Chang *et al.* [19] also infer that several proteins may undergo post-translational modifications under O_2-deprived conditions. Besides this reprogramming of gene expression, metabolic (e.g., switch to a fermentative pathway [34]) and structural (e.g., aerenchyma formation [35, 36]) changes occur during flooding. The genes and proteins induced by anaerobic stress in maize seedlings have extensively been reviewed previously [37–39].

CYTOSOLIC Ca^{2+} PERTURBATIONS PRECEDE ANOXIC GENE EXPRESSION IN PLANTS

The genes encoding the ANPs (e.g., *adh1*) are rapidly turned on even by mild hypoxia and turned off rapidly upon reoxygenation [14, 19, 40, 41]. Such a response implicates a fast and precise O_2-sensing system operating in plant cells. However, until recently, the pathway leading to the perception and transduction of low O_2 signals remained a "Black Box". The work initiated in our laboratory has identified a Ca^{2+}-mediated pathway of anoxic gene induction as well as post-anoxic seedling survival in maize cells and seedlings [42–44]. Work from several laboratories indicates that a similar pathway may operate in other plant species such as *Arabidopsis*, rice and wheat [6, 45–49]. Oxygen deprivation is also accompanied by cytosolic acidification [50]. The transient changes in Ca^{2+} and H^+ that follow cell stimulation are immediately recognized even at sub-micromolar levels, amplified and finally translated into long-lasting biochemical and physiological responses by plant cells [51–54]. Deprivation of O_2 leads to disturbances in ionic balance of plant cells, reflecting energy depletion and membrane depolarization [50, 55]. We have shown that gene expression and physiological changes in response to O_2 deprivation are preceded and signaled by an elevation of cytosolic Ca^{2+} in maize seedlings and cultured cells [42, 43]. We developed a single cell system to monitor cytosolic Ca^{2+} changes and an assay to measure anoxic responses in terms of expression of marker genes and seedling or cell survival [42, 43]. Using calcium channel antagonists and analyzing cytosolic free calcium ($[Ca]_i$) changes, we demonstrated that, calcium acts as a transducer of low O_2 signals both in suspension cultured cells and intact seedlings [42, 43]. Ruthenium red, a Ca^{2+} channel blocker, repressed the activation of the anoxia-inducible genes and impaired the post-anoxic survival of seedlings and cells [42, 43]. Ca^{2+}, when supplied along with ruthenium red, allowed both anoxic gene expression and survival, showing that Ca^{2+} acted very early in the adaptive response to anoxia. In maize suspension-cultured cells, O_2 depletion caused an immediate (within minutes) increase in $[Ca]_i$ and this was reversible within a few seconds of reoxygenation. Ruthenium red decreased the resting levels of $[Ca]_i$ and blocked the anoxic Ca^{2+} elevation. Caffeine, which induced an elevation of $[Ca]_i$ under aerobic conditions, caused an increase in ADH activity under normoxia.

Furthermore, we showed that Ca^{2+} influx was not necessary for the anoxia-induced $[Ca]_i$ elevation or early anoxic responses, indicating that the Ca^{2+}-rise observed under anoxia was due to mobilization of the ion from intracellular stores [42]. The origin of the calcium signal was traced, to elucidate the nature and intracellular location of the oxygen sensor. Being the primary site of oxygen consumption and also an important target of RR action, it was thought the mitochondrion might serve as a Ca^{2+} store in response to anoxia in maize cells. Confocal analysis using compartment-specific Ca^{2+} probes showed that the Ca^{2+} signal probably originates in mitochondria [44]. Isolated wheat mitochondria also respond to anoxia by immediate release of Ca^{2+} into the medium [49]. The early anoxic release of Ca^{2+} from mitochondria in intact maize cells may not be due to passive leakage of the ion, since it was not preceded by the depolarization of mitochondria [44], although isolated wheat mitochondria appear to behave differently (see [49]). Prolonged anoxia (>30 min) leads to a loss of mitochondrial membrane potential even in intact maize cells, which may be responsible for further Ca^{2+} release [44].

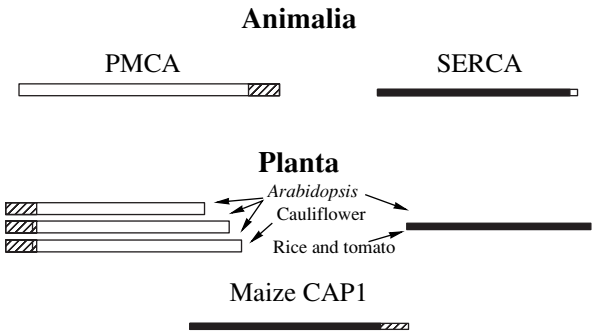
While the role of plasma membrane redox systems and associated second messengers also need to be examined, our findings placed mitochondria at the center of oxygen sensing. The elucidation of how O_2 deprivation initiates the Ca^{2+} release from mitochondria, may indicate exactly where the changes in O_2 levels are sensed in the cell. Since oxygen is more diffusive than any potential signal molecule that has to traverse the cellular membranes, anoxia may be first sensed at the mitochondrial electron transport chain, where O_2 would no longer be available as an electron acceptor. However, in view of the sensitivity of gene expression changes even to mild alterations in the O_2 availability [41], i.e., the genes are induced at much higher concentrations than the $K_m(O_2)$ of cytochrome a_3, a low affinity system could be a more appropriate sensor (such as a component of the plasma membrane redox system). In *Arabidopsis*, O_2 deprivation stimulates a Rop (RHO-like [Ras (oncogene that causes *rat sarcoma*) RHO-molog] small monomeric G protein of plants) signal transduction pathway. Activation of a Ca^{2+}-dependent NAD(P)H oxidase located either on the plasma membrane or the inner mitochondrial membrane results in H_2O_2 production and *ADH* gene activation [48]. Thus, the Ca^{2+} released from mitochondria under O_2 deprivation may link the metabolic changes occurring in the cytosol (and mitochondria) with the changes initiated in the nucleus. Consistent with this, large anoxia-induced changes in the nuclear localized Ca^{2+} levels were observed in maize cells [44], which may be a prelude to the chromatin changes that occur in anoxic maize cells [41].

Ionic Homeostasis as an Integral Part of Ca^{2+}-Mediated Anoxia Signaling

Our recent results indicate that cells use the cytosolic Ca^{2+} changes not only for triggering downstream signaling components but also to establish ionic homeostasis. The metabolic and structural adaptations to O_2 deprivation are known to precede transcriptional activation/repression of genes, translation of specific mRNA species and post-translational modification of proteins. Our interest is to understand how Ca^{2+} participates or leads to these events. It is notable that products of several genes induced by O_2 deprivation are Ca^{2+} or calmodulin-binding proteins (see [6, 56]). In addition, we believe that the perturbations in cytosolic Ca^{2+} may also mediate immediate adaptations needed for short-term cell survival. Under energy-deprived conditions, an imminent danger to cells (the organism, as well) is an unregulated traffic of ions. For example, a continued elevation of Ca^{2+} or decline in pH, if unattenuated, is not only detrimental in the long run but may also impair the capacity of cells to mount the adaptive responses. Therefore, ionic homeostasis is undoubtedly a key component of the cellular adaptive pathway under stress. We examined if Ca^{2+}-perturbations regulate the process of ionic equilibration in O_2-deprived cells. In maize root tip cells, cytosolic pH sharply decreases in response to anoxia from pH 7.5 to 6.9 within the first 10 min, but then quickly stabilizes at 7.1 over the next 10–15 minutes [57, 58]. One of the mechanisms proposed to revert the pH decline is the activation of proton-consuming enzymes, such as malic enzyme or glutamate decarboxylase [39, 50, 59], although the actual players have not yet been clearly worked out. Our contention that $[Ca]_i$ changes could be important in the establishment of the pH-state was reinforced by the discovery that plant glutamate decarboxylases (GADs) have a distinct ability of interacting with calmodulin (CAM) [60, 61]. Furthermore, GAD activity in many species exhibits a sharp pH optimum of 5.8 with little activity at or near neutral pH in the absence of CAM [61]. Therefore, a pH- and/or CAM-dependent activation and its significant ability to consume protons [60], make GAD a candidate regulator of cytosolic pH under anoxia. GAD activity was in fact shown to be induced in carrot protoplasts, by limiting the supply of O_2 for two hours [62]. Hence, we focused our studies on the activation of GAD as an adaptive response and a focal point of interaction between pH and calcium changes, in maize roots. A rapid induction of GAD activity as well as an increased association of this activity with CAM-containing protein complexes was observed in maize roots, within minutes of anoxic treatment. Furthermore, Ca^{2+}/CAM antagonists abolished the activity *in vitro*, indicating that CAM-association may be needed for the activation of GAD under anoxia (Subbaiah and Sachs, unpublished). The kinetics of GAD activation soon after the onset of anoxia, coincided with the time course of pH stabilization in maize root tips reported previously [58]. Transcriptional induction of GAD in O_2-deprived *Arabidopsis* root cultures also follows similar kinetics as revealed by recent microarray analysis, i.e., GAD mRNA increased during the first 30 min of low oxygen treatment but declined subsequently [6]. Several maize clones that are similar to GAD from other plant species were identified in a maize EST (Expressed Sequence Tag) database (http://www.zmdb.iastate.edu/), indicating that a gene family may encode this enzyme in maize. These clones were obtained and after confirmatory sequencing analysis were identified as putative maize GAD cDNAs. Sequence comparison indicated that GAD is encoded by at least 3 genes in maize (Subbaiah and Sachs, unpublished). RNA gel blot analysis showed that only one of them was inducible in maize roots during early anoxia, whereas the other two were actually repressed. Furthermore, the transcript levels for the non-inducible clones showed a greater abundance in the axis portion (one cm away from the tip) of the primary root in three-day old seedlings (Subbaiah and Sachs, unpublished). We have also examined the distribution of ESTs among various maize libraries that were made use of. The majority of the hits were found in the libraries that were made from young and meristematic tissues (leaf and tassel primordia, early embryo, and anthers) that are expected to be rich in mitochondria and show intense respiratory activity (Subbaiah and Sachs, unpublished).

Fine-tuning the Ca^{2+} signal. Efflux transporters (transporters that remove Ca^{2+} from the cytoplasm) play an equally important role as the influx transporters (transporters that allow Ca^{2+} into the cytoplasm) in Ca^{2+} signaling. Besides preventing the rise of Ca^{2+} to cytotoxic levels, high-affinity transporters such as Ca^{2+}-ATPases, modulate stimulus-induced Ca^{2+} signals. In view of the versatility of Ca^{2+} to transduce a number of stimuli, it is imperative that Ca^{2+} signals are patterned in a stimulus-specific manner. In coordination with the influx transporters, Ca^{2+} pumps not only orchestrate stimulus-specificity to Ca^{2+} signals but also propagate them [63]. One objective of our research is to unravel Ca^{2+}-transporters involved in the regulation of anoxic Ca^{2+} signal. To this end, we have isolated a cDNA clone, CAP1, from anoxic maize roots. CAP1 encodes a Ca^{2+}-ATPase and is induced during the first 4–6 h of anoxia [64]. This clone shares amino acid sequence identity with the animal Ca^{2+}-ATPases located on the endoplasmic reticulum (SERCAs or ER-type calcium pumps). However, maize CAP1 differs from other ER-type Ca^{2+}-pumps in that it has a calmodulin-binding domain at its carboxy-terminus. In animal cells, the plasma membrane located Ca^{2+} ATPase (PMCA) is distinctly different from SERCAs in sequence as well as regulation and possesses the CAM-binding domain at its C-terminus. The CAP1 cDNA complemented yeast mutants defective in Ca^{2+} pumps. The CAP1 protein from yeast membranes formed a phosphorylated intermediate characteristic of Ca^{2+}-ATPases and supported CAM-stimulated Ca^{2+} transport [64]. In plants, CAM-regulated Ca^{2+} pumps reported thus far have greater

sequence similarity to animal PMCAs but have their CAM-binding domain at the N-terminus. CAP1, by resembling SERCAs in its overall sequence and yet possessing a C-terminal CAM-binding domain, represents a novel chimeric calcium pump (Fig. 1), that may be involved in Ca^{2+}/CAM-mediated signaling pathways in maize roots. The *cap1* gene in maize encodes a low-abundant mRNA that is induced only during early anoxia, among other abiotic stresses tested [64]. This indicated the potential involvement of CAP1 in imparting anoxia-specificity to the Ca^{2+}-signal. The low abundance of CAP1 transcripts coincided with the scarce amounts of cognate protein in maize microsomes, indicating a tight regulation of CAP1 expression. Furthermore, calmodulin-regulation of Ca^{2+} transport capacity suggests the involvement of CAP1 product in attenuating cytosolic Ca^{2+} rise in a feedback manner during cell stimulation. A stringent regulation of Ca^{2+} efflux should allow the Ca^{2+}-dependent signaling processes to continue without the cell attaining cytotoxic levels of free Ca^{2+}. Induction of CAP1 transcripts in maize roots only during the first few hours of anoxia indicates such a regulation of Ca^{2+} homeostasis in the O_2-deprived maize cells [64].



Maize CAP1 is a novel chimeric calcium pump. CAP1 although more similar in its sequence to the animal endoplasmic reticulum-located calcium ATPases (SERCAs, indicated by a narrow dark bar), possesses a calmodulin-binding domain at the C-terminus (bar with crossed lines) similar to canonical plasma-membrane located pumps of animal cells (PMCAs, indicated by an unfilled wide bar and a bar with crosslines). Calmodulin-regulated Ca^{2+} pumps reported from other plant species are similar in their sequence to PMCAs but possess the CAM-binding domain at the N-terminus. They are also variable in their sizes. Thus, maize CAP1 is also unique among plant Ca^{2+}-ATPases.

Ca^{2+}-Dependent Posttranslational Regulation of Sucrose Synthase under Anoxia

One common mechanism that cells use to rapidly decipher and amplify the $[Ca]_i$ changes is reversible protein phosphorylation. Addition or removal of phosphate can lead to changes in the activation status, catalytic activity, or cellular localization of effector proteins [65, 66]. These changes, in turn, can lead to transient alterations in gene expression and metabolism or even long-lasting modifications in the plant form and function [67–70].

We have recently shown that an anaerobically-induced polypeptide, sucrose synthase1 (SH1), is post-translationally regulated by phosphorylation, and this regulation is among the early responses that culminate in the death of primary root tip in anoxic maize seedlings [30]. Sucrose synthase (SS) is a unique enzyme with an ability to mobilize sucrose into diverse pathways that are critical in structural (e.g., cellulose or callose biosynthesis), storage (starch synthesis) and metabolic (e.g., glycolysis) functions of plant cells [71]. It is encoded by two genes in maize, *sh1* (encoding SH1) and *sus1* (encoding SUS1). The *sh1* gene is expressed mostly in the developing endosperm, whereas *sus1* is expressed in many plant parts including aleurone and basal part of the developing endosperm. The *sh1* gene is induced by anoxia both at transcriptional and translational levels (ANP87) [13]. The *sus1* gene is only mildly induced by anoxia. Although the double mutants in SS have been shown to be less tolerant to anoxia [72], the contribution of SH1, i.e., the anoxia-inducible isoform, to anoxia tolerance had not been previously examined. Our results indicate that the differential regulation of the two genes at transcriptional and translational levels extends into the post-translational level, with potent effects on adaptation to anoxia and endosperm development [30].

Analysis of Ca^{2+}-dependent changes in protein phosphorylation under anoxia indicated that the SH1 isoform of sucrose synthase was phosphorylated at increased rates in maize roots experiencing 2-h anoxia. In contrast, during prolonged anoxia, the protein was under-phosphorylated, and by 48 h, most of the protein existed in an unphosphorylated form. In seedlings submerged for 2 h or longer, a part of the SH1 became associated with the microsomal fraction [30]. The membrane localization of SH1 increased with the duration of anoxia, but was confined only to the root tip. This preceded an extensive induction of callose formation and other symptoms of root tip death (e.g., induction of nuclear DNA breakage; Subbaiah and Sachs, unpublished). Consistent with the Ca^{2+} dependence of SS phosphorylation [73], EGTA addition to the submergence buffer led to an increased dephosphorylation as well as membrane localization of SH1 and greater callose accumulation. On the other hand, Ca^{2+} addition decreased the proportion of membrane-bound SH1 and callose deposits [30]. Thus, these results corroborated two earlier observations: (1) sucrose synthase is functionally associated with glucan synthases in the plasma membrane [74] and (2) phosphorylation status of SS may determine its partitioning between soluble and membrane fractions [75]. Furthermore, our genetic analysis suggested that this response was isoform-specific in that *sh1* mutants maintained SS phosphorylation and had low amounts of callose deposits in the root tip even under prolonged anoxia. This correlated with

the superior anoxia tolerance of *sh1* mutants to that of the nonmutants [30]. Our studies thus indicate a functional divergence of SS isoforms due to a differential post-translational regulation, in that, SUS1, existing mostly as a soluble form, may supply hexoses to glycolysis, while SH1, being distributed to both soluble and membrane fractions, contributes to the biosynthesis of cell wall polymers as well. Such a dichotomy is also consistent with the proposed roles of SS isoforms in the developing maize endosperm (see [76]).

Hypoxia-Induced Aerenchyma or Anoxia-Induced Root Tip Death: Ca^{2+}-Regulated Cell Death as a Means of Survival under Oxygen Deprivation

The essence of stress adaptation is redirecting scarce resources to the maintenance of essential sinks as well as activation of adaptive pathways, while disinvesting in non-essential sinks and pathways. Being endowed with multiple growing points, plants have a unique ability to eliminate superfluous tissues/organs under stress and regenerate them if favorable conditions demand. O_2-deprived maize roots exhibit two such regulated cell or tissue-death pathways. These two pathways although clearly distinct in their symptomology as well as the location of their occurrence in the root, appear to be regulated by Ca^{2+}.

Inner cortical cell layers of the primary or nodal roots are selectively killed under hypoxia (i.e., partial submergence when only the roots were submerged), leading to aerenchyma formation [77]. This selective cell death not only reduces the demand for O_2 but more importantly, enhances root porosity and facilitates oxygen diffusion from the exposed plant parts into the submerged ones. Aerenchyma formation requires the presence of oxygen and occurs 3–4 cm behind the tip [78]. This allows the adaptation of root tip to the localized anoxia [79], and the prolonged survival of the seedlings. The nature and regulation of cell death during aerenchyma formation have been the subject of recent studies [77, 78, 80]. These studies indicate that the aerenchyma formation occurs by a genetically programmed cell death (PCD), that is clearly dependent on Ca^{2+} (reviewed in [77]). Cytohistological data indicate that the hypoxically-induced PCD may not be entirely similar to the canonical apoptic pathway of animal cells, but partly resembles the cytoplasmic or necrotic death [80].

Root-tip death. Under complete submergence, maize seedlings exhibit another cell death process that also appears to have an adaptive significance. Although prolonged anoxia ultimately kills the entire seedling, different tissues of an individual plant differ in their tolerance [81, 82]. Maize root tips that are not hypoxically-acclimated are very sensitive to anoxia and die within a few hours [50, 81]. Root tips are composed of tightly packed tissues with few, if any, intercellular spaces and hence suffer from restricted gaseous diffusion. Consequently, in flooded seedlings, root tip death may be a natural consequence of oxygen starvation and the attendant repression of substrate transport. Considerable attention has been paid to strategies/mechanisms that prolong the anoxia tolerance of the primary root tip in young maize seedlings, as the tip of the primary root is considered to be very important for seedling establishment (for a review, see [83]). On the other hand, we proposed that under severe anoxia, when energy generation is extremely limiting, the loss of metabolically intensive tissues such as the root-tip might prolong the survival of the shoot and the root axis. The facilitated survival of these two organs during submergence may increase the chances of seedling recovery after reoxygenation. We tested this proposal recently and results indicate that the root tip indeed acts as a dispensable and non-functional sink in anoxic seedlings [30, 84].

The time course of primary root tip death in submerged maize seedlings was followed using the post-anoxic development of visible necrotic symptoms and uptake of Evans Blue as criteria. Anoxia for 48 h or longer led to the death of the root tip [84]. If the seedlings were reaerated prior to 48 h, the root tip death did not occur. However, cell death indicators such as callose development, DNA nicking and induction of hydrolytic activities were observed to occur much earlier than 48 h ([30] and unpublished data). Mitochondria isolated from wheat roots release cytochrome *c* under anoxia [49]. These observations indicated that the death process, although initiated before 24 h, became irreversible by 48 h of anoxia. The necrosis extended into the root axis during post-anoxic recovery, leading to the mortality of 30–50% of the seedlings. Excision of the root tip (detipping) before anoxia led to a 30–40% greater recovery of seedlings from stress injury. Unlike in the case of intact seedlings with slow and progressive death of the root tip, de-tipped seedlings show less shoot and root damage resulting in a rapid emergence of leaves as well as lateral roots, after re-aeration [84]. Our data also indicate that the dying root tip of intact seedlings releases diffusible death-inducing factors into the submergence buffer, as indicated by the acceleration of seedling death when submergence buffer was reused. Preliminary analysis indicates that these factors are proteinaceous in nature ([85] and unpublished data). Therefore, a reprogramming of root tip death to make it occur early during anoxia, may provide a definite adaptive advantage to maize seedlings to anoxic stress. In *Arabidopsis*, the whole root system is dispensable for hypoxic tolerance of the seedlings; in fact, derooted seedlings fared better under O_2 deprivation [82]. In maize, the primary root axis is helpful (in quickly producing a functional root system), if not essential, for the postanoxic recovery of seedlings. However, the survival of the shoot meristem is critical for the post-anoxic re-growth and autotrophic life of the seedling.

Anoxia-induced protease (AIP) in root tip death. To identify potential regulators of the cell death process, changes in protease activities were analyzed in the root

tissues. Cysteine and serine proteases have been implicated in the cell death/injury induced by abiotic, biotic or developmental signals in plants [86–88] (for a review, see [89]).

Different species of proteases, both soluble and membrane-bound, are induced or suppressed during various durations of anoxic stress and reoxygenation in roots of three-day old dark-grown maize seedlings [84]. The major aerobic proteases are suppressed after 6-h anoxia and new enzymes are detected both in soluble and membrane fractions. Upon reoxygenation, the aerobic activities reappear and the anoxic enzymes persist for at least 24 h after seedlings are reaerated. We observed a soluble enzyme that became detectable after 12-h anoxia. This enzyme increases with time and accounts for the major proteolytic activity in roots of 48-h-long submerged seedlings [84]. Protein synthesis inhibitor studies indicate it to be a newly synthesized enzyme under anoxia (AIP). AIP activity runs as a 22–25 kD complex in SDS-PAGE. Ca^{2+} is required for the renaturation and proteolytic activity of the enzyme and inhibitor sensitivities indicated that AIP is a cysteine protease. Detipping caused a decrease in AIP activity. Thus, the appearance of AIP activity in the root tip before 24-h submergence was spatially and temporally associated with the initiation of the root tissue death [84].

In addition to AIP activity, XET1 mRNA is also induced in maize roots by anoxia (apparently by a different mechanism than its hypoxic induction [18]) and may be involved in the root-tip death process. Besides its proposed role in cell wall loosening in growing tissues, XET is associated with cell-wall hydrolysis and cell lysis. For example, increased XET activity was shown to have a temporal correlation with ethylene-induced fruit ripening and softening [90].

Root tip death under anoxia: programmed cell death or necrosis? Cell death is a basic biological process important in the regulated development of multicellular organisms and in their responses to stress. Animal cells show two fundamentally different modes of death, namely apoptosis (or PCD) and necrosis. The most relevant distinction between the two types of death is the early preservation of membrane integrity in apoptosis, whereas a rapid release of intracellular constituents occurs in the case of necrosis. Therefore, necrosis can presumably be dangerous, while apoptic response is an adaptive mechanism to dispose cells without compromising the rest of the organism. Nevertheless, more and more evidence points out that apoptosis and necrosis represent just extreme ends of wide range of possible morphological and biochemical deaths. Root tip death is preceded by SH1 relocation, DNA nicking, induction of AIP as well as callose, indicating that the process, to some extent, is autonomous (and a programmed event) [30, 84 and unpublished data]. On the other hand, the death of root tip cells is accompanied by the acidification of the cytosol [50] as well as the external medium and an extracellular release of diffusible cytotoxins ([85] and unpublished data). Therefore, root tip death in nature may be a less cell-autonomous but more of a necrotic process. Our detipping experiments suggest that an acceleration of the process as well as making it more cell-autonomous (i.e., pushing the process more towards PCD) would provide a definite advantage during postanoxic recovery of maize seedlings. Indeed, some maize genotypes appear to have evolved an accelerated root tip death as a genetically-controlled flooding tolerance mechanism [91].

We also propose that calcium may moderate the cell death in the root tip in maintaining it as a cell-autonomous process. This is indicated by the requirement of Ca^{2+} for the induction as well as activity of AIP and potential role of Ca^{2+} in the anoxic induction of XET (see [92]). Depletion of Ca^{2+} in the flooding buffer suppresses the induction of AIP, promotes the dephosphorylation of SS and accentuates callose induction in the root tip as well as in the axis, while extending the necrosis into the axis of the primary root [30]. Recent studies using isolated wheat root mitochondria suggest that the cytochrome *c* release from mitochondria (a characteristic feature of PCD in animal cells) is a Ca^{2+}-dependent process under anoxia [49].

CONCLUSIONS

Anoxia is one of the most important abiotic stresses encountered by most higher organisms. The anaerobic stress-response of maize offers an opportunity to characterize the regulatory components of a family of twenty genes that are coordinately expressed. The anaerobically-induced proteins appear to be encoded by a set of genes whose expression is stimulated by a deprivation of oxygen, a condition that would occur in nature during flooding. Regulation of protein synthesis under anaerobiosis appears to occur at multiple levels. We have characterized several genes involved in the anaerobic-response and provided some insight into a few components of the signal transduction pathway. Our goal has been to understand how maize perceives the changes in external O_2 concentration and adapts its growth and metabolism in short and long time scales. To this end, we have demonstrated that Ca^{2+} acts as a key transducer of changes in O_2 availability. We also present evidence for the role of Ca^{2+} in a rapid attainment of ionic homeostasis (essential for a sustained activation of downstream signaling components) as well as in long-term adaptations that involve regulated cell death.

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REFERENCES

1. Suszkiw, J., After the Flood: Satellites Show Damage to Midwest Farmlands, *Agric. Res.*, 1994, vol. 42, pp. 20–21.
2. Quebedeaux, B. and Hardy, R.W.F., Reproductive Growth and Dry Matter Production of *Glycine max* (L.) Merr. in Response to Oxygen Concentration, *Plant Physiol.*, 1975, vol. 55, pp. 102–107.
3. Kuang, A., Crispi, M., and Musgrave, M.E., Control of Seed Development in *Arabidopsis thaliana* by Atmospheric Oxygen, *Plant Cell Environ.*, 1998, vol. 21, pp. 71–78.
4. Blasiak, J. and Musgrave, M.E., Varietal Differences in Locular Gas Composition in Developing Fruit of Sweet and Hot Peppers, *Capsicum* spp., and Evidence for Divergent Diffusion Pathways, *J. Hort. Sci. Biotech.*, 2002, vol. 77, pp. 432–437.
5. Sachs, M.M., Freeling, M., and Okimoto, R., The Anaerobic Proteins of Maize, *Cell*, 1980, vol. 20, pp. 761–767.
6. Klok, E.J., Wilson, I.W., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R., and Dennis, E.S., Expression Profile Analysis of the Low-Oxygen Response in *Arabidopsis* Root Cultures, *Plant Cell*, 2002, vol. 14, pp. 2481–2494.
7. Kelley, P.M. and Tolan, D.R., The Complete Amino Acid Sequence for the Anaerobically Induced Aldolase from Maize Derived from cDNA Clones, *Plant Physiol.*, 1986, vol. 82, pp. 1076–1080.
8. Laszlo, A. and St Lawrence, P., Parallel Induction and Synthesis of PDC and ADH in Anoxic Maize Roots, *Mol. Gen. Genet.*, 1983, vol. 192, pp. 110–117.
9. Kelley, P.M., Maize Pyruvate Decarboxylase mRNA Is Induced Anaerobically, *Plant Mol. Biol.*, 1989, vol. 13, pp. 213–222.
10. Lal, S.K., Lee, C., and Sachs, M.M., Differential Regulation of Enolase during Anaerobiosis in Maize, *Plant Physiol.*, 1998, vol. 118, pp. 1285–1293.
11. Kelley, P.M. and Freeling, M., Anaerobic Expression of Maize Glucose Phosphate Isomerase, *J. Biol. Chem.*, 1984, vol. 259, pp. 673–677.
12. Russell, D.A. and Sachs, M.M., The Maize Glyceraldehyde-3-Phosphate Dehydrogenase Gene Family: Organ-Specific Expression and Genetic Analysis, *Mol. Gen. Genet.*, 1991, vol. 229, pp. 219–228.
13. Springer, B., Werr, W., Starlinger, P., Bennett, D.C., Zokolica, M., and Freelin, M., The *shrunken* Gene on Chromosome 9 of *Zea mays* L. Is Expressed in Various Plant Tissues and Encodes an Anaerobic Protein, *Mol. Gen. Genet.*, 1986, vol. 205, pp. 461–468.
14. Freeling, M., Simultaneous Induction by Anaerobiosis or 2.4-D of Multiple Enzymes Specified by Two Unlinked Genes: Differential *Adh1–Adh2* Expression in Maize, *Mol. Gen. Genet.*, 1973, vol. 127, pp. 215–227.
15. Vogel, J. and Freeling, M., An Anaerobic Gene, Which Encodes an Apparently Nonglycolytic Protein, Shares Sequence Homology with *Mu1.7* and *Mu* Related Sequence-A, *Maize Genet. Cooper. News Lett.*, 1992, vol. 66, pp. 21–22.
16. Peschke, V.M. and Sachs, M.M., Characterization and Expression of Anaerobically Induced Maize Transcripts, *Plant Physiol.*, 1994, vol. 104, pp. 387–394.
17. Saab, I.N. and Sachs, M.M., Complete cDNA and Genomic Sequence Encoding a Flooding-Responsive Gene from Maize (*Zea mays* L.) Homologous to Xyloglucan *Endo*transglycosylase, *Plant Physiol.*, 1995, vol. 108, pp. 439–446.
18. Saab, I.N. and Sachs, M.M., A Flooding-Induced Xyloglucan *Endo*-Transglycosylase Homolog in Maize Is Responsive to Ethylene and Associated with Aerenchyma, *Plant Physiol.*, 1996, vol. 112, pp. 385–391.
19. Chang, W.W.P., Huang, L., Shen, M., Webster, C., Burlingame, A.L., and Roberts, J.K.M., Patterns of Protein Synthesis and Tolerance of Anoxia in Root Tips of Maize Seedlings Acclimated to a Low-Oxygen Environment, and Identification of Proteins by Mass Spectrometry, *Plant Physiol.*, 2000, vol. 122, pp. 295–318.
20. Ferl, R.J., Brennan, M., and Schwartz, D., *In vitro* Translation of Maize ADH: Evidence for the Anaerobic Induction of mRNA, *Biochem. Genet.*, 1980, vol. 18, pp. 681–691.
21. Rowland, L.J. and Strommer, J.N., Anaerobic Treatment of Maize Roots Affects Transcription of *Adh1* and Transcript Stability, *Mol. Cell Biol.*, 1986, vol. 6, pp. 3368–3372.
22. Bailey-Serres, J., Kloeckener-Gruissem, B., and Freeling, M., Genetic and Molecular Approaches to the Study of the Anaerobic Response and Tissue Specific Gene Expression in Maize, *Plant Cell Environ.*, 1988, vol. 11, pp. 351–357.
23. Dennis, E.S., Walker, J.C., Llewellyn, D.J., Ellis, J.G., Singh, K., Tokuhisa, J.G., Wolstenholme, D.R., and Peacock, W.J., The Response to Anaerobic Stress: Transcriptional Regulation of Genes for Anaerobically Induced Proteins, *Environmental Stress in Plants*, Cherry, J.H., Ed., New York: Springer-Verlag, 1989, pp. 231–245.
24. Webster, C., Gaut, R.L., Browning, K.S., Ravel, J.M., and Roberts, J.K.M., Hypoxia Enhances Phosphorylation of Eukaryotic Initiation Factor 4A in Maize Root Tips, *J. Biol. Chem.*, 1991, vol. 266, pp. 23341–23346.
25. Russell, D.A. and Sachs, M.M., Differential Expression and Sequence Analysis of the Maize Glyceraldehyde-3-Phosphate Dehydrogenase Gene Family, *Plant Cell*, 1989, vol. 1, pp. 793–803.
26. Russell, D.A. and Sachs, M.M., Protein Synthesis in Maize during Anaerobic and Heat Stress, *Plant Physiol.*, 1992, vol. 99, pp. 615–620.
27. de Vetten, N.C. and Ferl, R.J., Characterization of a Maize G-box Binding Factor That Is Induced by Hypoxia, *Plant J.*, 1995, vol. 7, pp. 589–601.
28. Manjunath, S. and Sachs, M.M., Molecular Characterization and Promoter Analysis of the Maize Cytosolic Glyceraldehyde 3-Phosphate Dehydrogenase Gene Family and Its Expression during Anoxia, *Plant Mol. Biol.*, 1997, vol. 33, pp. 97–112.
29. Manjunath, S., Williams, A.J., and Bailey-Serres, J., Oxygen Deprivation Stimulates Ca^{2+}-Mediated Phosphorylation of mRNA Cap-Binding Protein eIF4E in Maize Roots, *Plant J.*, 1999, vol. 19, pp. 21–30.
30. Subbaiah, C.C. and Sachs, M.M., Altered Patterns of Sucrose Synthase Phosphorylation and Localization Precede Callose Induction and Root Tip Death in Anoxic

Maize Seedlings, *Plant Physiol.*, 2001, vol. 125, pp. 585–594.

31. Bailey-Serres, J. and Freeling, M., Hypoxic Stress-Induced Changes in Ribosomes of Maize Seedling Roots, *Plant Physiol.*, 1990, vol. 94, pp. 1237–1243.
32. Bailey-Serres, J. and Dawe, K., Both 5' and 3' Sequences of Maize ADH1 mRNA Are Required for Enhanced Translation under Low-Oxygen Conditions, *Plant Physiol.*, 1996, vol. 112, pp. 685–695.
33. Perez-Mendez, A., Aguilar, R., Briones, E., and Sanchez De, J.E., Characterization of Ribosomal Protein Phosphorylation in Maize Axes during Germination, *Plant Sci.*, 1993, vol. 94, pp. 71–79.
34. Kennedy, R.A., Rumpho, M.E., and Fox, T.C., Anaerobic Metabolism in Plants, *Plant Physiol.*, 1992, vol. 84, pp. 1204–1209.
35. Drew, M.C., Jackson, M.B., and Giffard, S., Ethylene-Promoted Adventitious Rooting and Development of Cortical Air Spaces (Aerenchyma) in Roots May Be Adaptive Responses to Flooding in *Zea mays* L., *Planta*, 1979, vol. 147, pp. 83–88.
36. Drew, M.C., Saglio, P.H., and Pradet, A., Larger Adenylate Energy Charge and ATP/ADP Ratios in Aerenchymatous Roots of *Zea mays* in Anaerobic Media as a Consequence of Improved Internal Oxygen Transport, *Planta*, 1985, vol. 165, pp. 51–58.
37. Sachs, M.M., Molecular Genetic Basis of Metabolic Adaptation to Anoxia in Maize and Its Possible Utility for Improving Tolerance of Crops to Soil Waterlogging, *NATO ASI Series, Vol. 16: Interacting Stresses on Plants in a Changing Environment*, Jackson, M.B. and Black, C.R., Eds., Berlin: Springer-Verlag, 1993, pp. 375–393.
38. Sachs, M.M., Gene Expression in Maize during Anoxia, *Stress Induced Gene Expression in Plants*, Basra, A.S., Ed., Chur, Switzerland: Harwood Academic, 1994, pp. 87–102.
39. Sachs, M.M., Subbaiah, C.C., and Saab, I.N., Anaerobic Gene Expression and Flooding Tolerance in Maize, *J. Exp. Bot.*, 1996, vol. 47, pp. 1–15.
40. Wignarajah, K. and Greenway, H., Effect of Anaerobiosis on Activities of Alcohol Dehydrogenase and Pyruvate Decarboxylase in Roots of *Zea mays*, *New Phytol.*, 1976, vol. 77, pp. 575–584.
41. Paul, A.-L. and Ferl, R.J., *Adh1* and *Adh2* Regulation, *Maydica*, 1991, vol. 36, pp. 129–134.
42. Subbaiah, C.C., Bush, D.S., and Sachs, M.M., Elevation of Cytosolic Calcium Precedes Anoxic Gene Expression in Maize Suspension Cultured Cells, *Plant Cell*, 1994, vol. 6, pp. 1747–1762.
43. Subbaiah, C.C., Zhang, J., and Sachs, M.M., Involvement of Intracellular Calcium in Anaerobic Gene Expression and Survival of Maize Seedlings, *Plant Physiol.*, 1994, vol. 105, pp. 369–376.
44. Subbaiah, C.C., Bush, D.S., and Sachs, M.M., Mitochondrial Contribution to the Anoxic Ca^{2+} Signal in Maize Suspension-Cultured Cells, *Plant Physiol.*, 1998, vol. 118, pp. 759–771.
45. Sedbrook, J.C., Kronebush, P.J., Borisy, C.G., Trewavas, A.J., and Masson, P.H., Transgenic AEQUORIN Reveals Organ-Specific Cytosolic Ca^{2+} Responses to Anoxia in *Arabidopsis thaliana* Seedlings, *Plant Physiol.*, 1995, vol. 111, pp. 243–257.
46. Aurisano, N., Bertani, A., and Reggiani, R., Involvement of Calcium and Calmodulin in Protein and Amino Acid Metabolism in Rice Roots under Anoxia, *Plant Cell Physiol.*, 1995, vol. 36, pp. 1525–1529.
47. Chung, H.-J. and Ferl, R.J., *Arabidopsis* Alcohol Dehydrogenase Expression in Both Shoots and Roots Is Conditioned by Root Growth Environment, *Plant Physiol.*, 1999, vol. 121, pp. 429–436.
48. Baxter-Burrill Bailey-Serres, J., Yang, Z., Springer, P.S., and Bailey-Serres, J., RopGAP4-Dependent Rop GTPase Rheostat Control of *Arabidopsis* Oxygen Deprivation Tolerance, *Science*, 2002, vol. 296, pp. 2026–2028.
49. Virolainen, E., Blokhina, O., and Fagerstedt, K., Ca^{2+}-Induced High Amplitude Swelling and Cytochrome *c* Release from Wheat (*Triticum aestivum* L.) Mitochondria under Anoxic Stress, *Ann. Bot.*, 2002, vol. 90, pp. 509–516.
50. Roberts, J.K.M., Callis, J., Wemmer, D., Walbot, V., and Jardetzky, O., Mechanism of Cytoplasmic pH Regulation in Hypoxic Maize Root Tips and Its Role in Survival under Hypoxia, *Proc. Natl. Acad. Sci. USA*, 1984, vol. 81, pp. 3379–3383.
51. Knight, H., Trewavas, A.J., and Knight, M.R., Cold Calcium Signaling in *Arabidopsis* Involved Two Cellular Pools and a Change in Calcium Signature after Acclimation, *Plant Cell*, 1996, vol. 8, pp. 489–503.
52. Fasano, J.M., Swanson, S.J., Blancaflor, E.B., Dowd, P.E., Kao, T.H., and Gilroy, S., Changes in Root Cap pH Are Required for the Gravity Response of the *Arabidopsis* Root, *Plant Cell*, 2001, vol. 13, pp. 907–921.
53. Bush, D.S., Calcium Regulation in Plant Cells and Its Role in Signaling, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1995, vol. 46, pp. 7–13.
54. Sanders, D., Brownlee, C., and Harper, J.F., Communicating with Calcium, *Plant Cell*, 1999, vol. 11, pp. 691–706.
55. Buwalda, F., Thompson, C.J., Steigner, W., Barrett-Lennard, E.G., Gibbs, J., and Greenway, H., Hypoxia Induces Membrane Depolarization and Potassium Loss from Wheat Roots but Does Not Increase Their Permeability to Sorbitol, *J. Exp. Bot.*, 1988, vol. 39, pp. 1169–1183.
56. Day, I.S., Reddy, V.S., Ali, G.S., and Reddy, A.S.N., Analysis of EF-Hand-Containing Proteins in *Arabidopsis*, *Genome Biol.*, 2002, vol. 3, pp. 56.1–56.24.
57. Saint-Ges, V., Roby, C., Bligny, R., Pradet, A., and Douce, R., Kinetic Studies of the Variations of Cytoplasmic pH, Nucleotide Triphosphates (^{31}P-NMR) and Lactate during Normoxic and Anoxic Transitions in Maize Root Tips, *Eur. J. Biochem.*, 1991, vol. 200, pp. 477–482.
58. Fox, G.G., McCallan, N.R., and Ratcliffe, R.G., Manipulating Cytoplasmic pH under Anoxia: A Critical Test of the Role of pH in the Switch from Aerobic to Anaerobic Metabolism, *Planta*, 1995, vol. 195, pp. 324–330.
59. Ratcliffe, R.G., *In vivo* NMR Studies of the Metabolic Responses of Plant Tissues to Anoxia, *Ann. Bot.*, 1997, vol. 79 (Suppl. A), pp. 39–48.
60. Snedden, W.A., Arazi, T., Fromm, H., and Shelp, B.J., Calcium/Calmodulin Regulation of Soybean Glutamate Decarboxylase, *Plant Physiol.*, 1995, vol. 108, pp. 543–549.
61. Shelp, B.J., Bown, A.W., and McLean, M.D., Metabolism and Functions of Gamma-Aminobutyric Acid, *Trends Plant Sci.*, 1999, vol. 4, pp. 446–452.

62. Carroll, A.D., Fox, G.G., Laurie, S., Phillips, R., Ratcliffe, R.G., and Stewart, G.R., Ammonium Assimilation and the Role of γ-Aminobutyric Acid in pH Homeostasis in Carrot Cell Suspensions, *Plant Physiol.*, 1994, vol. 106, pp. 513–520.

63. Camacho, P. and Lechleiter, J.D., Increased Frequency of Calcium Waves in *Xenopus laevis* Oocytes That Express a Calcium ATPase, *Science*, 1993, vol. 260, pp. 226–229.

64. Subbaiah, C.C. and Sachs, M.M., Maize *cap1* Encodes a Novel SERCA-Type Calcium ATPase with a Calmodulin-Binding Domain, *J. Biol. Chem.*, 2000, vol. 275, pp. 21678–21687.

65. Kim, J., Blackshear, P.J., Johnson, J.D., and McLaughlin, S., Phosphorylation Reverses the Membrane Association of Peptides That Correspond to the Basic Domains of MARCKS and Neuromoduli, *Biophys. J.*, 1994, vol. 67, pp. 227–237.

66. Huber, J.L.A. and Huber, S.C., Role and Regulation of Sucrose-Phosphate Synthase in Higher Plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1996, vol. 47, pp. 431–444.

67. Ferreira, P.C., Hemerly, A.S., and Van Montagu, M., A Protein Phosphatase 1 from *Arabidopsis thaliana* Restores Temperature Sensitivity of a *Schizosaccharomyces pombe cdc25^{ts}/wee1^{-}* Double Mutant, *Plant J.*, 1993, vol. 4, pp. 81–87.

68. Maurel, C., Kado, R.T., Guern, J., and Chrispeels, M.J., Phosphorylation Regulates the Water Channel Activity of the Seed-Specific Aquaporin α-Tip, *EMBO J.*, 1995, vol. 14, pp. 3028–3035.

69. Stone, J.M., Trotochaud, A.E., Walker, J.C., and Clark, S.E., Control of Meristem Development by CLAVATA1 Receptor Kinase and Kinase-Associated Protein Phosphatase Interactions, *Plant Physiol.*, 1998, vol. 117, pp. 1217–1235.

70. Fankhauser, C., Yeh, K.-C., Lagarias, J.C., Zhang, H., Elic, T.D., and Chory, J., PKS1, a Substrate Phosphorylated by Phytochrome That Modulates Light Signaling in *Arabidopsis*, *Science*, 1999, vol. 284, pp. 1539–1541.

71. Ruan, Y.-L., Chourey, P.S., Delmer, D.P., and Perez-Grau, L., The Differential Expression of Sucrose Synthase in Relation to Diverse Patterns of Carbon Partitioning in Developing Cotton Seed, *Plant Physiol.*, 1997, vol. 115, pp. 375–385.

72. Ricard, B., Van Toai, T., Chourey, P., and Saglio, P., Evidence for the Critical Role of Sucrose Synthase for Anoxic Tolerance of Maize Roots Using a Double Mutant, *Plant Physiol.*, 1998, vol. 116, pp. 1323–1331.

73. Huber, S.C., Huber, J.L., Liao, P.C., Gage, D.A., McMichael, R.W., Jr., Chourey, P.S., Hannah, L.C., and Koch, K.E., Phosphorylation of Serine-15 of Maize Leaf Sucrose Synthase. Occurrence *in vivo* and Possible Regulatory Significance, *Plant Physiol.*, 1996, vol. 112, pp. 793–802.

74. Amor, Y., Haigler, C., Johnson, S., Wainscott, M., and Delmer, D.P., A Membrane-Associated Form of Sucrose Synthase and Its Potential Role in Synthesis of Cellulose and Callose in Plants, *Proc. Natl. Acad. Sci. USA*, 1995, vol. 92, pp. 9353–9357.

75. Winter, H., Huber, J., and Huber, S.C., Membrane Association of Sucrose Synthase: Changes during the Graviresponse and Possible Control by Protein Phosphorylation, *FEBS Lett.*, 1997, vol. 420, pp. 151–155.

76. Chourey, P.S., Taliercio, E.W., Carlson, S.J., and Ruan, Y.-L., Genetic Evidence That the Two Isozymes of Sucrose Synthase Present in Developing Maize Endosperm Are Critical, One for Cell Wall Integrity and the Other for Starch Biosynthesis, *Mol. Gen. Genet.*, 1998, vol. 259, pp. 88–96.

77. Drew, M.C., He, C.-J., and Morgan, P.W., Programmed Cell Death and Aerenchyma Formation in Roots, *Trends Plant Sci.*, 2000, vol. 5, pp. 123–127.

78. He, C.-J., Morgan, P.W., and Drew, M.C., Enhanced Sensitivity to Ethylene in Nitrogen- or Phosphate-Starved Roots of *Zea mays* L. during Aerenchyma Formation, *Plant Physiol.*, 1992, vol. 98, pp. 137–142.

79. Gibbs, J., de Bruxelle, D., Armstrong, W., and Greenway, H., Evidence for Anoxic Zones in 2–3 mm Tips of Aerenchymatous Maize Roots under Low O_2 Supply, *Aust. J. Plant Physiol.*, 1995, vol. 22, pp. 723–730.

80. Gunavardena, A.H.L.A.N., Pearce, D.M., Jackson, M.B., Hawes, C.R., and Evans, D.E., Characterization of Programmed Cell Death during Aerenchyma Formation Induced by Ethylene or Hypoxia in Roots of Maize (*Zea mays* L.), *Planta*, 2001, vol. 212, pp. 205–214.

81. Johnson, J., Cobb, B.G., and Drew, M.C., Hypoxic Induction of Anoxia Tolerance in Root Tips of *Zea mays*, *Plant Physiol.*, 1989, vol. 91, pp. 837–841.

82. Ellis, M.H., Dennis, E.S., and Peacock, W.J., *Arabidopsis* Roots and Shoots Have Different Mechanisms for Hypoxic Tolerance, *Plant Physiol.*, 1999, vol. 119, pp. 57–64.

83. Drew, M.C., Cobb, B.G., Johnson, J.R., Andrews, D., Morgan, P.W., Jorda, W., and He, C.J., Metabolic Acclimation of Root Tips to Oxygen Deficiency, *Ann. Bot.*, 1994, vol. 74, pp. 281–286.

84. Subbaiah, C.C., Kollipara, K., and Sachs, M.M., A Ca^{2+}-Dependent Cysteine Protease Is Associated with Anoxia-Induced Root Tip Death in Maize, *J. Exp. Bot.*, 2000, vol. 51, pp. 721–730.

85. Subbaiah, C.C., Kollipara, K., and Sachs, M.M., Potential Involvement of Maize AIP in the Anoxia-Induced Death of the Root Tip, *Abstracts of 41st Annual Maize Genetics Conference, Lake Geneva, WI, March 11–14, 1999*, p. 98.

86. Williams, J., Bulman, M., Huttl, A., Phillips, A., and Neil, S., Characterization of a cDNA from *Arabidopsis thaliana* Encoding a Potential Thiol Protease Whose Expression Is Induced Independently by Wilting and Abscisic Acid, *Plant Mol. Biol.*, 1994, vol. 25, pp. 259–270.

87. Stroeher, V.L., Maclagan, J.L., and Good, A.G., Molecular Cloning of a *Brassica napus* Cysteine Protease Gene Inducible by Drought and Low Temperature Stress, *Physiol. Plant.*, 1997, vol. 101, pp. 389–397.

88. Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., and Levine, A., The Involvement of Cysteine Proteases and Protease Inhibitor Genes in the Regulation of Programmed Cell Death in Plants, *Plant Cell*, 1999, vol. 11, pp. 431–443.

89. Hadfield, K.A. and Bennett, A.B., Programmed Senescence of Plant Organs, *Cell Death Differentiation*, 1997, vol. 4, pp. 662–670.

90. Redgwell, R.J. and Fry, S.C., Xyloglucan *Endo*transglycosylase Activity Increases during Kiwifruit (*Actinidia deliciosa*) Ripening, *Plant Physiol.*, 1993, vol. 103, pp. 1399–1406.
91. Zeng, Y., Avigne, W.T., and Koch, K.E., Rapid Repression of Maize Invertase by Low Oxygen. Invertase/Sucrose Synthase Balance, Sugar Signaling Potential and Seedling Survival, *Plant Physiol.*, 1999, vol. 121, pp. 599–608.
92. Polisensky, D.H. and Braam, J., Cold-Shock Regulation of the *Arabidopsis* TCH Genes and the Effects Modulating Intracellular Calcium Levels, *Plant Physiol.*, 1996, vol. 111, pp. 1271–1279.